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BASIC SCIENCE: OBSTETRICS

The origin of fetal sterols in second-trimester amniotic fluid: endogenous synthesis or maternal-fetal transport?

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OBJECTIVE: Cholesterol is crucial for fetal development. To gain more insight into the origin of the fetal cholesterol pool in early human pregnancy, we determined cholesterol and its precursors in the amniotic fluid of uncomplicated, singleton human pregnancies.

STUDY DESIGN: Total sterols were characterized by gas chromatography–mass spectrometry in the second-trimester amniotic fluid of 126 healthy fetuses from week 15 until week 22.

RESULTS: The markers of cholesterol biosynthesis, lanosterol, dihydrolanosterol, and lathosterol, were present in low levels until the 19th week of gestation, after which their levels increased strongly. β -sitosterol, a marker for maternal-fetal cholesterol transport, was detectable in the amniotic fluid. The total cholesterol levels increased slightly between weeks 15 and 22.

CONCLUSION: Our results support the hypothesis that during early life the fetus depends on maternal cholesterol supply because endogenous synthesis is relatively low. Therefore, maternal cholesterol can play a crucial role in fetal development.

Key words: cholesterol biosynthesis, embryology, maternal-fetal cholesterol transport

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Cholesterol is the most important sterol in humans. Its role in membrane fluidity and as a precursor of bile acids and steroid hormones has been discussed extensively. In addition to these important functions in adult mammals, cholesterol is also crucial for embryonic development. It activates the sonic hedgehog (Shh) proteins and propagates their signaling; after being modified by addition of cholesterol. Shh sets off a cascade of events in target cells, leading to the activation and repression of genes by transcrip-

tion factors in the Gli family. This Shh-Gli pathway is known to be one of the fundamental signal transduction pathways in mammals, responsible for the development of different organ systems, including the heart.¹ Because of these indispensable structural and regulatory functions, the availability of cholesterol must be guaranteed throughout embryonic and fetal development.²

Although fetal tissues synthesize cholesterol, we and others have shown that maternal cholesterol also contributes

substantially to the fetal cholesterol pool in animal models.³⁻⁶ However, only a few studies have addressed this in humans. Vuorio et al⁷ discovered that, just after birth, plant sterol concentrations in cord blood of healthy newborns were 40-50% of the maternal levels, indicative for active maternal-fetal sterol transport. Furthermore, a specific maternal lipid profile, resulting in a more rapid maternal-fetal cholesterol transport, tends to result in a milder phenotype of Smith-Lemli-Opitz syndrome (SLOS), a disorder of cholesterol biosynthesis.⁸ This suggests that the maternal cholesterol supply is of great importance during early life and that disturbances in lipid transport can have adverse effects on the fetal development.

Before uteroplacental circulations are established, maternal-fetal nutrient exchange of high-molecular-weight molecules (eg, proteins and lipids) takes place through the yolk sac, which is connected to the ventral part of the embryo and the main blood circulation through the vitelline veins. Molecules from the mother leak easily into the exocoelomic cavity because of the loose mesenchymal layer of early placental tissue.⁹ The yolk sac absorbs these molecules and excretes them

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The authors report no conflict of interest.

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into the fetal circulation.¹⁰ Various sterol transporter proteins are expressed on both the yolk sac and the early placenta, suggesting active transport of these molecules to fetal tissues.^{3,11,12} The yolk sac in humans regresses around week 8 of gestation when the placenta takes over its role in nutrient exchange.

In addition to active transport by the vitelline veins, the amniotic fluid (AF) surrounding the embryo may also play an important role in maternal-fetal nutrient exchange.¹³ Several lipoprotein receptors and enzymes involved in lipoprotein uptake, such as the low-density lipoprotein receptor and lipoprotein lipase, are found on the apical surface of the amniotic membrane at term, suggesting that lipoproteins from the amniotic fluid or maternal circulation are taken up by the amnion and could therefore serve as a transporter for fetal lipids.¹⁴ From animal studies we know that changes in maternal diet directly influence the AF composition.¹⁵ However, because most of the AF consists of fetal bioproducts,¹⁶ the AF seems to be an interesting medium to study with regard to the origin of fetal sterols.

Despite the important roles of cholesterol in fetal development and of maternal-fetal cholesterol transport, the origin of the fetal cholesterol pool in the first half of a human pregnancy is still unclear. This is mainly because of the obviously limited accessibility of the human fetus for transport studies. Because umbilical functions are not routinely performed in prenatal diagnosis, the amniotic fluid surrounding the fetus can be used as an alternative medium for measuring endogenous synthesis and maternal-fetal cholesterol transport. Amniotic fluid samples are readily available from amniocentesis samples. However, the few human studies that are performed to analyze the concentration of different sterols in AF are small in sample size with fewer than 100 cases and detailed information about the sterol concentrations related to gestational age is lacking.¹⁷

While we were analyzing our data, Amaral et al¹⁸ published a paper in which they reported measurements of different sterol precursors in AF. Our study significantly adds to their data, which is fur-

ther explained in the Comment section of the article.

In this study, we used sterol concentrations in amniotic fluid as markers for fetal sterol turnover. The cholesterol precursors, lanosterol, dihydrolanosterol, lanosterol, and desmosterol, are all important biochemical markers of cholesterol biosynthesis. We analyzed these sterols to determine the relative cholesterol synthesis rates in the second trimester of human pregnancies. Furthermore, we determined β -sitosterol levels in AF, one of the most important plant sterols. Because humans are not capable of synthesizing plant sterols and because plant sterols are transported in a similar way to cholesterol, we assumed this would be a valid marker to measure maternal-fetal cholesterol transport.

Because cholesterol is of crucial importance for heart development through its role in Shh signaling, we analyzed concentrations of cholesterol and its precursors, together with plant sterols in the AF of pregnancies complicated by isolated fetal congenital heart anomalies to test our hypothesis that maternal-fetal cholesterol transport is important for providing the cholesterol needed for proper fetal development.

We had 2 aims: first, to investigate the origin of the fetal cholesterol pool in second-trimester amniotic fluid of healthy pregnancies (is it derived from synthesis [represented by increased precursor levels] or from maternal-fetal transport [represented by increased β -sitosterol levels])? Second, because cholesterol is especially important for fetal heart development, we compared the levels of different sterols and their precursors in pregnancies with an isolated fetal heart defect with the levels in pregnancies without heart anomalies, matched for gestational age.

MATERIALS AND METHODS

Biological samples

Sample group 1

We retrospectively selected AF samples from 126 singleton pregnancies with a normal birth outcome in 2003 until 2010 at the University Medical Center Groningen. Amniocentesis was performed

mainly if there was an increased risk for aneuploidy because of a positive prenatal screening or because of increased maternal age.

Directly after amniocentesis the AF samples were stored at -20°C . Women were asked whether surplus fluid, not needed for diagnosis, could be used for research. This conforms with the hospital code of good practice for surplus material of the Dutch Consortium of Scientific Associations. Ethics approval was given by the University Medical Center Groningen Ethics Committee.

Information on birth outcome (with informed consent) was available for all pregnancies and obtained from hospital records, from Eurocat Northern Netherlands (a population-based birth defects registry that covers 80% of the university's hospital population) and from population-based perinatal registration records. Samples were organized according to gestational age from week 15 until week 22. Numbers for each week of gestational age were as follows: week 15, $n = 14$; week 16, $n = 20$; week 17, $n = 41$; week 18, $n = 18$; week 19, $n = 7$; week 20, $n = 11$; to week 21, $n = 9$; to week 22, $n = 6$. Unfortunately, we could not make groups of equal size for each week because we selected the samples retrospectively.

Sample group 2

We retrospectively selected AF of 40 singleton pregnancies that were complicated by a congenital heart anomaly of the fetus, which was diagnosed prenatally and confirmed after birth. Cases with multiple congenital anomalies were excluded. Because amniocentesis was performed after the diagnosis of congenital heart anomalies by prenatal ultrasound scan (routinely performed in The Netherlands around week 20 of gestation), we could include samples only from gestational week 19 until week 23. Groups were too small to divide the heart anomalies into different cardiac subgroups.

Sample group 3

We selected additional control samples of children and fetuses prenatally diagnosed with the monogenetic disorder os-

TABLE 1

Sterol concentrations in 126 amniotic fluid samples of healthy fetuses

Variable	Week 15 (n = 14)	Week 16 (n = 20)	Week 17 (n = 41)	Week 18 (n = 18)	Week 19 (n = 7)	Week 20 (n = 11)	Week 21 (n = 9)	Week 22 (n = 6)
Cholesterol	54.30 ± 16.51	53.90 ± 16.92	51.30 ± 14.34	56.84 ± 13.24	74.15 ± 26.02	66.62 ± 25.19	79.60 ± 36.51	66.05 ± 36.22
Lanosterol	0.010 ± 0.003	0.009 ± 0.004	0.004 ± 0.002	0.008 ± 0.004	0.019 ± 0.015	0.105 ± 0.192	0.133 ± 0.104	0.103 ± 0.027
Dihydrolanosterol	0.002 ± 0.001	0.002 ± 0.002	0.003 ± 0.002	0.008 ± 0.004	0.016 ± 0.016	0.021 ± 0.009	0.055 ± 0.036	0.071 ± 0.033
Lathosterol	0.117 ± 0.049	0.095 ± 0.038	0.136 ± 0.065	0.222 ± 0.095	0.528 ± 0.446	0.890 ± 0.442	1.958 ± 1.482	1.689 ± 0.267
Desmosterol	0.450 ± 0.156	0.447 ± 0.204	0.476 ± 0.141	0.532 ± 0.118	0.569 ± 0.250	0.418 ± 0.139	0.461 ± 0.255	0.423 ± 0.179
β -sitosterol	0.165 ± 0.088	0.140 ± 0.077	0.084 ± 0.024	0.091 ± 0.025	0.173 ± 0.075	0.110 ± 0.058	0.220 ± 0.115	0.097 ± 0.023

Concentrations ($\mu\text{mol/L}$) of cholesterol, lathosterol, sitosterol, desmosterol, dihydrolanosterol, and lanosterol were determined in 126 amniotic fluid samples of healthy fetuses from gestational weeks 15 to 22.

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teogenesis imperfecta, a skeletal disorder that is not related to cholesterol disturbances in the mother or the child. All the cases were matched to 1 control from the same gestational week. If there was more than 1 control, they were pooled.

Sample group 4

Three fresh AF samples were obtained from women at 16 weeks of gestation who underwent amniocentesis for increased risk of aneuploidy because of increased maternal age. These were used to evaluate cholesterol fractions in cells and free fluid after centrifuging for 10 minutes at 2000 rpm without freezer storage.

Reagents, standards, and sample preparation

Twenty-five microliters of cholestane (milligrams per milliliter) and 10 μL of epicoprostanol were added as internal standards to 500 μL of AF samples. Alkaline hydrolysis was performed adding 1.5 mL 10% ethanolic NaOH (1M) followed by heating at 60°C for 60 minutes. The samples were diluted with 0.5 mL of deionized water, and the lipids were extracted with 3 mL of cyclohexane. The lipids were derivatized by adding 50 μL of n-decane and 10 μL of trimethylsilyl ether at 70°C for 60 minutes and analyzed by gas chromatography–mass spectrometry (GC-MS) according to the methods described by Thelen et al.¹⁹

Statistical analysis

Statistical analysis was performed using SPSS software (version 18.0 for Windows; SPSS Inc, Chicago, IL). Spear-

man's correlation test was used to compare sterol distributions as well as to evaluate the correlation of the parameters gestational age and sterol concentrations. We used the Mann-Whitney *U* test to compare sterol distributions between cases and controls for each gestational age and in total.

RESULTS

Sterol concentrations in normal pregnancies

Our data for sterol levels in the AF of 126 healthy fetuses followed a nonlinear distribution. Concentrations for each sterol at each gestational week (with 95% confidence intervals) are shown in Table 1. The total cholesterol levels were slightly increased between week 15 and 22 (Figure 1), although there was a very high variation between 16.3 and 140.5 $\mu\text{mol/L}$.

Figure 1 shows the correlations between the different sterol precursors and gestational age.

A strong, statistically significant correlation between dihydrolanosterol and lathosterol concentrations with gestational age was obtained with a correlation coefficient close to 1 ($P < .001$). These sterol precursors were detectable in very low levels until the 19th week of gestation, after which their levels increased strongly. For lanosterol we found a moderately positive correlation, showing the same pattern as dihydrolanosterol and lathosterol (Figure 1). β -sitosterol was detectable in significant amounts in AF and varied throughout the second trimester of pregnancy (Figure 1). The same pattern was also observed for

desmosterol (Figure 1) and for other relevant plant sterols and stanols (campesterol, stigmasterol and cholestanol) (data not shown).

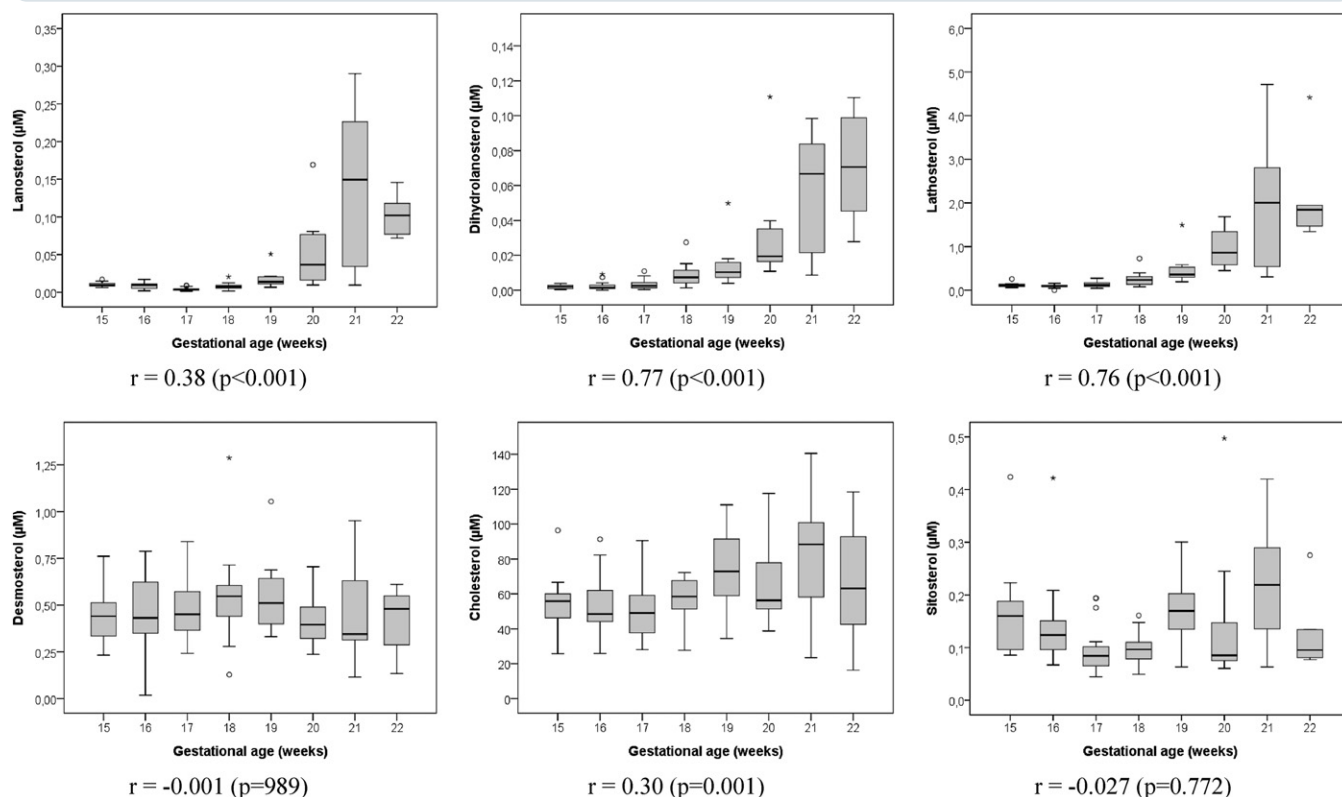
Desmosterol and β -sitosterol were both strongly correlated with total cholesterol levels (data not shown). For the sterol precursors lanosterol, dihydrolanosterol, and lathosterol, we found positive moderate correlations with total cholesterol concentrations. After correcting for total cholesterol concentration, dihydrolanosterol and lathosterol levels remained strongly correlated with gestational age; whereas, lanosterol remained moderate positively correlated with gestational age. Desmosterol and β -sitosterol showed a moderately negative correlation after correcting for total cholesterol ($P < .001$) (Table 2).

Sterol concentrations in congenital heart disease

We compared sterol concentrations in AF of fetuses with confirmed nonsyndromic congenital heart disease and age-matched controls (Figure 2). Lanosterol levels were significantly lower in the cases than in controls in weeks 21–22 ($P = .013$; $P = .04$). Concentrations for β -sitosterol were significantly lower in cases than in controls between weeks 19 and 20 ($P = .038$; $P = .016$). We did not find any statistically significant differences between cases and controls for dihydrolanosterol, lathosterol, or desmosterol (levels equally distributed in both cases and controls) or for total cholesterol concentrations.

FIGURE 1

Correlation between sterols and gestational age



Spearman correlation coefficients are shown for each graph. Asterisks and circles show outliers and extreme values, which were excluded in our calculations.

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COMMENT

We analyzed different sterols and their precursors in second-trimester human AF to investigate the origin of fetal cho-

lesterol. We found a significant increase of the cholesterol precursors lanosterol, dihydrolanosterol, and lathosterol from gestational week 19 onward, indicating increasing fetal synthesis from that moment in time. Our results suggest that during early life the fetus depends on maternal cholesterol as an exogenous source.

Amniotic fluid is a part of the fetal compartment, and therefore, lipids found in the AF belong to the fetal pool. Because human fetal tissues of uncomplicated pregnancies are not readily available, we propose here that amniotic fluid is a valuable alternative for studies on early cholesterol synthesis and maternal-fetal cholesterol transport.

There are 2 pathways of cholesterol synthesis in humans. One is the Bloch pathway via zymosterol and desmosterol, and the other is the Kandutsch-Russell pathway through lathosterol and 7-dehydrocholesterol.²⁰ Previous studies have shown that the first is active in

tissue of human brains between weeks 10 and 20 of gestation. However, the 2 studies that report this are very small in numbers and show contrary results.^{21,22} Because the few patients ($n = 7$) that are described with desmosterolosis, an autosomal recessive disorder caused by mutations in genes that code for proteins involved in the final step of the Bloch pathway, mainly show brain anomalies,²³ the role of desmosterol in the development of other organ systems is still under debate.

Our data showed that levels of desmosterol remained constant compared with the raised levels of the sterol precursors from the Kandutsch-Russell pathway. The almost constant total cholesterol concentrations, together with the increased sterol precursor concentrations, suggest an increased fetal cholesterol synthesis rate from week 19 onward. Thus, our data suggest that, in the first half of pregnancy, exogenous (maternal) cholesterol supply is sufficient to

TABLE 2

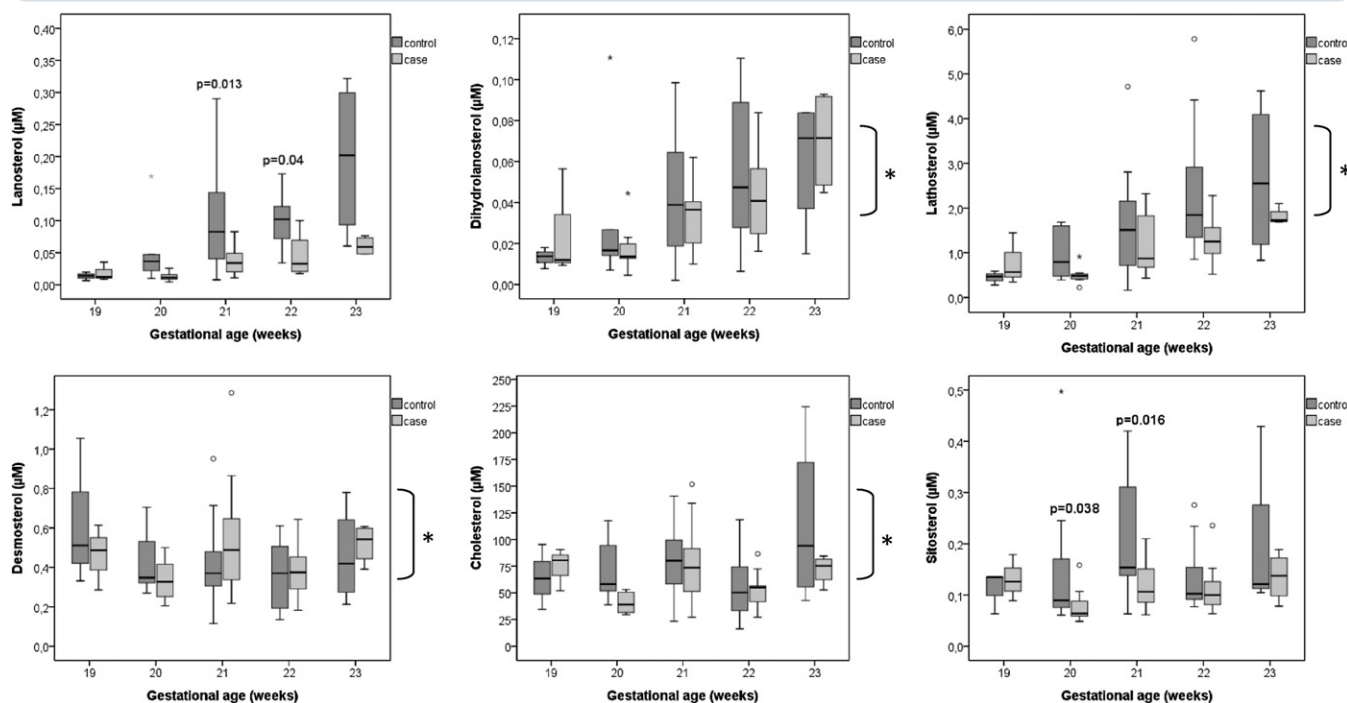
Correlation between the cholesterol ratios of different sterol precursors and gestational age

Variable	Spearman's correlation coefficient	P value
Lanosterol	0.33	< .001
Dihydrolanosterol	0.81	< .001
Lathosterol	0.78	< .001
Desmosterol	-0.297	.001
β-Sitosterol	-0.305	.001

Correlation between the cholesterol ratios of different sterol precursors and gestational age in second-trimester amniotic fluid of 126 healthy fetuses after correction for cholesterol concentrations.

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FIGURE 2
Sterol concentrations in congenital heart disease



Statistically significant P values of the Mann-Whitney U test are shown in the graphs. Asterisks and circles display outliers and extreme values, which were excluded in our calculations. There were no differences in total distribution of sterols according to gestational age between cases and controls (Mann-Whitney U test).

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fulfill the fetal demands, whereas from week 19 onward, fetal cholesterol synthesis via the Kandutsch-Russell pathway becomes increasingly important.

One limitation of our study is that, because of the retrospective study design, we could not measure maternal cholesterol concentrations in plasma and therefore could not calculate the maternal plasma to amniotic fluid ratio of total cholesterol.

Plant sterols are transported via membranes in a comparable way with cholesterol and are here used as markers for maternally derived cholesterol because their only source is the maternal diet. We found that β -sitosterol and other plant sterols remained constant throughout the second trimester of pregnancy, which indicates that the fetus mainly uses cholesterol from the mother for its cholesterol needs and premature biosynthesis.

Recently Amaral et al¹⁸ published a paper in which they reported measurements of different sterol precursors in AF. They

reported that levels of total cholesterol and its precursors, lathosterol, desmosterol, and 7-dehydrocholesterol, increase as gestation progresses. We could confirm their data only on lathosterol concentrations. 7-Dehydrocholesterol was not detectable in our samples. They concluded that fetal cholesterol synthesis increases from week 19 onward; however, this conclusion was based on absolute increased precursor levels only.

In contrast, we gained support for our hypothesis for an increased level of cholesterol synthesis on augmented ratios of precursor to cholesterol levels (Table 2). Measurements of absolute levels of sterols in AF are insufficient because of reported variations in the consistency of the fluid (ie, more diluted or more concentrated).²⁴

Our study also adds new data on lanosterol and dihydrolanosterol levels in amniotic fluid showing the same increase in concentration as gestation progresses and supporting the data on la-

thosterol. One important difference between our study and that of Amaral et al¹⁸ is that they analyzed cell-free AF, whereas we used complete AF samples. We found that the cell fraction accounts for about 25% of total sterols (data not shown).

Cells in the AF can be categorized into 3 different cell types: epitheloid type cells (E-type), amniotic fluid-specific type cells (AF-type), and fibroblastic type cells (F-type). The E-type cells are derived from fetal skin and urine, the AF-type from fetal membranes and placental trophoblasts, and the F-type from connective and mesenchymal tissues and dermal fibroblasts.^{17,25}

These studies underline the importance of analyzing the complete AF sample as cholesterol and sterol precursors are detectable in all 3 cell types and therefore contribute significantly to the fetal cholesterol pool.

In children with SLOS, a disorder of cholesterol biosynthesis, a lack of cholest-

terol in utero disturbs Shh signaling, resulting in various heart anomalies. We hypothesized that the same mechanism might play a role in nonsyndromic heart defects. To test this, we investigated differences in sterol concentrations in the amniotic fluid of children with and without a congenital heart anomaly. We did not find any consistent significant differences between cases and controls, indicating that sterol metabolism is not involved in the majority of cases of congenital heart anomalies. However, based on our data, it cannot be excluded that some cases of congenital heart anomalies are related to transport deficits (measured by β -sitosterol) and others to insufficient synthesis (represented by lanosterol) or even by a combination of those two.

Our sample size was not large enough to focus on relevant subgroups reported to be influenced by cholesterol and Shh, like atrioventricular septal defects, tetralogy of Fallot, atrial septal defects, outflow tract anomalies such as persistent truncus arteriosus, and anomalies of the pulmonary veins.²⁶⁻³¹ We encourage other researchers to investigate total cholesterol and its precursors in the amniotic fluid of children with cholesterol- and Shh-related heart defects without a diagnosis of SLOS, ideally in a multicenter study.

Our study provides new insights into the origin of fetal cholesterol in early pregnancy and new information on the sterol precursors lanosterol and dihydrolanosterol. Our results suggest that, during early life, the fetus depends on maternal cholesterol as an exogenous cholesterol source because the endogenous synthesis through the Kandutsch-Russell pathway is not yet established. Maternal cholesterol could therefore play a crucial role in fetal development because cholesterol drives important developmental signaling pathways.

This information is particularly important because maternal conditions and diseases that disturb cholesterol concentrations (ie, obesity and diabetes) are increasing rapidly among pregnant women. Although we could not find any differences in the AF of fetuses with and without a congenital heart anomaly in the gestational period studied, the avail-

ability of maternal cholesterol very early in embryogenesis might still be an important factor in fetal heart development. Future research should now focus on maternal-fetal cholesterol transport in the first trimester of pregnancy during embryogenesis. ■

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